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Mutational Alterations Affecting the Export Competence of a Truncated but Fully Functional Maltose-Binding Protein Signal Peptide

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The wild-type maltose-binding protein (MBP) signal peptide is 26 amino acids in length. A mutationally altered MBP signal peptide has been previously described that is missing one of the basic residues from the hydrophilic segment and seven residues from the hydrophobic core; however, it still facilitates MBP secretion to the periplasm at a rate and efficiency comparable to those of the wild-type structure. Thus, this truncated signal peptide (designated the R2 signal peptide) must retain all of the essential features required for proper export function. In this study, alterations were obtained in the R2 signal peptide that resulted in an export-defective MBP. For the first time, signal sequence mutations were obtained that resulted in the synthesis of a totally export-defective MBP. As was previously the case for the wild-type signal peptide, the introduction of either charged residues or helix-breaking proline residues adversely affected export function. Despite these similarities, the position of these alterations within the R2 signal peptide, their relative effects on MBP secretion and processing, and an analysis of the ability of various extragenic *prl* mutations to suppress the secretion defects provide additional insight into the minimal requirements for a functional MBP signal peptide.

The periplasmic maltose-binding protein (MBP) of *Escherichia coli*, the *malE* gene product, is initially synthesized with an amino-terminal signal peptide that is thought to be chiefly responsible for initiating the secretion of this protein while it is still a nascent chain attached to the ribosome (7, 22, 25). The MBP signal peptide exhibits features of signal peptides of both procaryotic and eucaryotic origin (18, 21, 28). It is 26 amino acids in length and has at least two distinct regions, the hydrophilic segment and the hydrophobic core (see Fig. 1). The first eight residues, of which three are basic, represent the hydrophilic segment. This is followed by the hydrophobic core, a region devoid of charged residues and predicted to assume an α-helical conformation (8). The last six residues of the signal peptide are believed to represent the recognition sequence for the processing enzyme, signal peptidase. For the MBP, the processing site is immediately preceded by the sequence Ala-Leu-Ala. The sequence Ala-X-Ala is considered the consensus processing site for nonlipoprotein signal peptides (21, 26–28). This signal peptidase recognition sequence may represent a third region of the signal peptide, distinct from the hydrophobic core (21, 29, 30).

A number of *malE* mutations have been described that result in an export-defective MBP as a result of alterations in the signal peptide (Fig. 1) (3, 5, 7; reviewed in reference 4). In mutants with such mutations, the MBP accumulates in the cytoplasm in its unprocessed, precursor form. In addition, a number of phenotypically Mal^+ revertants of *malE* signal sequence mutants have been isolated that improve the export efficiency of the MBP to various degrees (3, 24). In certain instances, the reversion mutation results in a new alteration in the MBP, either within the signal peptide or early in the mature protein, that serves to partially or totally suppress the original export defect. Such genetic studies have provided valuable information concerning the requirements for a functional MBP signal peptide. It is now clear that the overall hydrophobicity of the core and the secondary structure through this region are both essential features. Still, the various properties that contribute to a fully functional signal peptide cannot yet be delineated. Likewise, the exact role of the mature protein in the export process is not known.

One of the *malE* signal sequence mutations obtained, designated *malEΔ12–18*, removed residues 12 through 18 from the center of the hydrophobic core and resulted in an MBP that was almost totally export defective (Fig. 1); less than 1% of the total MBP synthesized was secreted into the periplasm and processed (3). A Mal^+ revertant of a *malEΔ12–18* strain was isolated that substituted leucine for arginine at residue 8 of the signal peptide (designated *malEΔ12–18-R2* and henceforth referred to as R2) (Fig. 1) (3). The MBP signal peptide synthesized by the R2 mutant was 7 residues shorter than the wild type and had one fewer basic residue in the hydrophilic segment. Interestingly, it facilitated MBP secretion and processing at a rate and efficiency comparable to those of the wild-type structure (3). It was proposed that the effect of the alteration in the R2 mutant was to extend the truncated hydrophobic core into the hydrophilic segment by eliminating the nearest basic residue.

Since the R2 signal peptide is significantly shorter than the wild-type signal peptide and yet must retain all of the essential features required for proper export function, it was of interest to investigate this structure in more detail. In this study, seven different mutational alterations were obtained in the R2 mutant that resulted in an export-defective MBP. Each of these represents a single amino acid substitution in the R2 signal peptide. In certain respects, these changes are

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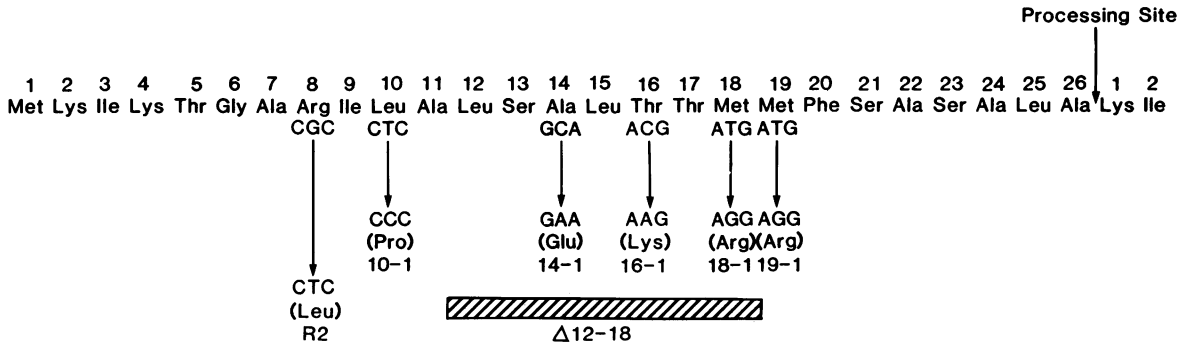

FIG. 1. Primary amino acid sequence of the wild-type MBP signal peptide and alterations resulting from various *malE* signal sequence mutations. The amino-terminal 28 residues of the wild-type pMBP are shown, including the entire signal peptide and the processing site. Single amino acid substitutions in the signal peptide that impair the export of this protein to the periplasm are shown immediately below the wild-type sequence. The responsible nucleotide alterations and allelic designations are also indicated. ▨, Residues removed from the signal peptide by the *malEΔ12–18* deletion mutation. We isolated a Mal$^+$ revertant of *malEΔ12–18*, designated *malEΔ12–18-R2* and referred to here as revertant R2, that substituted Leu for Arg at residue 8 of the signal peptide. See text for additional details and references.

analogous to ones previously obtained in the wild-type signal peptide (7). However, there are some interesting differences, and analysis of these mutants provides additional insight into the protein export process.

MATERIALS AND METHODS

Bacterial and bacteriophage strains, reagents, and genetic techniques. For the sake of clarity, strains are usually referred to in the text by their relevant genotypes, rather than by specific strain designations. All of the strains used in this study are isogenic derivatives of the *E. coli* K-12 strain MC4100: F$^-$ *ΔlacU169 araD139 rpsL150 thi flbB5301 deo-7 ptsF25 relA1* (9). The construction of *malE prlA* and *malE prlD* double mutants by using the generalized transducing phage P1 was performed as previously described (2). The *malE-lacZ* fusion phage λp72-47 was previously described (6). The minimal medium used was M63 (19) supplemented with the appropriate carbon sources at a final concentration of 0.2%. Maltose tetrazolium indicator agar was prepared as described previously (19). [^{35}S]methionine (translation grade; 1,154 Ci/mmol) was obtained from New England Nuclear Corp. Rabbit anti-MBP serum has been described previously (12). Electrophoresis reagents and Kodak XAR film were obtained from Bethesda Research Laboratories, Inc., and Eastman Kodak Co., respectively. Standard genetic techniques were performed as described by Miller (19).

Isolation of mutational alterations in the R2 signal peptide. We isolated a derivative of λp72-47 harboring a nonpolar *malE* deletion, designated *malEΔ323*, that removed DNA sequences encoding residue 7 of the MBP signal peptide through residue 89 of the mature MBP (V. A. Bankaitis and P. J. Bassford, Jr., unpublished results). Strain MC4100 lysogens of this phage are normally maltose-resistant (Mal^r) and Lac$^+$. Since this phage is *Δatt* and strain MC4100 and its derivatives are *Δlac*, integration of the phage genome into the *E. coli* chromosome requires recombination between their homologous *mal* DNA regions (5). By using strain MC4100 *malEΔ12–18-R2* (3), we obtained a rare lysogen of λp72-47 *malEΔ323* that exhibited a Mal$^-$ Lac$^+$ phenotype. In this case, integration of the phage DNA into the chromosome must have occurred via a single crossover event between the 3′ endpoint of the *malEΔ323* deletion and the *malE-lacZ* fusion point. The end result was a lysogen in which the *malEΔ323* deletion had been recombined into the chromosomal *malE* gene. At the same time, the R2 signal peptide-coding region, all of which falls under the *malEΔ323* deletion, was recombined into the *malE-lacZ* hybrid gene carried on the fusion phage. (A completely analogous reciprocal recombination event is illustrated in reference 5.) Phage λp72-47-R2 was subsequently recovered from this lysogen by heat induction.

Phage λp72-47-R2 was used to lysogenize strain MC4100 *malEΔ12–18-R2*. This construction, designated strain RL638, served to prevent the generation of new genotypes by any recombinational events between homologous *malE* regions present on the phage genome and chromosome. Strain RL638 exhibited a Mal^s Lac$^+$ phenotype. Mal^r Lac$^+$ derivatives of strain RL638 were isolated as previously described (5). Linkage of the mutation responsible for the Mal^r Lac$^+$ phenotype to the *malE-lacZ* fusion was established as previously described (5).

Presumptive signal sequence mutations isolated on phage λp72-47-R2 were recombined into the chromosomal *malE* gene by one of two methods. First, the plaque-purified phages recovered from each Mal^r Lac$^+$ mutant were used to lysogenize strain MC4100 *malEΔ323* at 30°C. The lysogens were then plated at 42°C, and Mal$^+$ survivors were selected. In such survivors, excision and subsequent curing of the prophage must have occurred in such a manner as to replace the *malEΔ323* deletion in the chromosome with the homologous region of the *malE-lacZ* hybrid gene carried on the phage genome, which includes the MBP signal peptide-coding region. Transfer of the putative signal sequence mutation to the chromosome was confirmed by marker rescue experiments, i.e., relysogenization with λp72-47-R2 resulted in a high frequency of Mal^r Lac$^+$ colonies.

As it turned out, this approach could not be used to construct the phenotypically Mal$^-$ signal sequence mutants CC19, CC21 and CC23 (see Results). The mutations harbored in each of these strains were recombined from the fusion phage on which they were originally isolated into the chromosomal *malE* gene of strain MC4100, with screening for Mal$^-$ colonies on maltose tetrazolium indicator agar, by the method previously described (5).

DNA sequencing. DNA sequence analyses of the signal sequence mutations obtained in this study were performed as previously described (3).

Radiolabeling, immune precipitation, SDS-PAGE, and autoradiography. Cells were grown in glycerol minimal me-

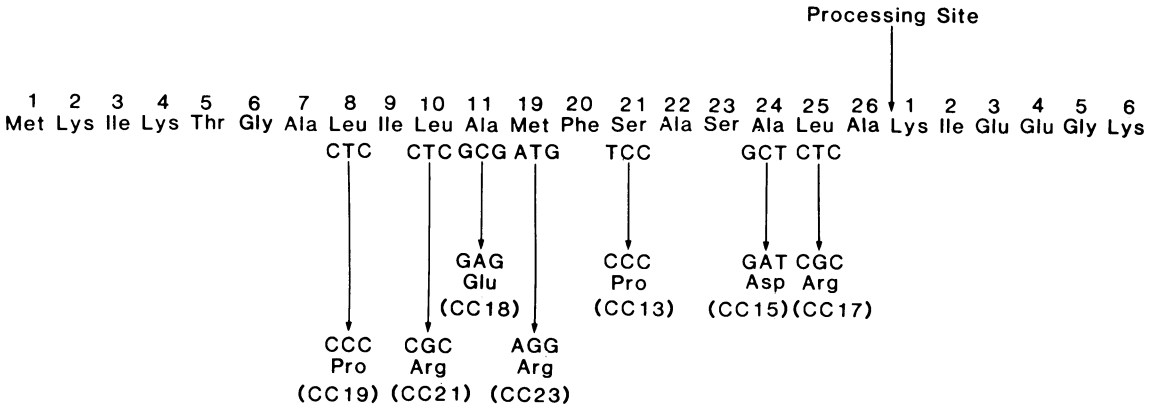

FIG. 2. Mutational alterations in the R2 signal peptide. The primary amino acid sequence of the truncated R2 signal peptide, including the R2-encoded alteration at position 8, and the first six residues of the mMBP are shown. Amino acid substitutions resulting from seven unique mutations in the R2 signal peptide coding region are indicated. The changes in the nucleotide sequence and the corresponding designations for each mutation are also given.

dium to mid-log phase and induced for *mal* regulon expression by the addition of 0.2% maltose to the culture medium. At 45 min later, cells were radiolabeled with [^{35}S]-methionine, and the MBP was immune precipitated from solubilized cell extracts by the procedures previously described (14). Immune precipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as previously described (17). Pulse-chase studies were also performed as previously described (23).

RESULTS

Isolation of mutational alterations in the R2 signal peptide. Since the R2 signal peptide facilitates MBP export with virtually wild-type efficiency, it was anticipated that mutations resulting in an export-defective R2 signal peptide could be isolated by using the same selection scheme previously used for the wild-type MBP signal peptide. For the latter, such mutations had been obtained by using the *malE-lacZ* gene fusion 72-47. It was found that signal sequence alterations that adversely affected the attempted export of the MalE-LacZ hybrid protein from the cytoplasm relieved the overproduction lethality resulting from its high-level synthesis (5–7, 17). The R2 signal sequence coding region was genetically recombined in *cis* to the 72-47 gene fusion carried on a specialized transducing phage, λp72-47 (6), as described in Materials and Methods. Presumptive signal sequence mutants were then isolated by selecting Mal^r Lac$^+$ derivatives. As expected, these Mal^r Lac$^+$ isolates had acquired new mutations that proved to be linked to the hybrid gene. For each of 10 independently obtained mutants chosen for further study, the mutation responsible for the Mal^r Lac$^+$ phenotype was recombined from the fusion phage into the chromosomal *malE* gene of an *E. coli* strain harboring the *malEΔ12–18-R2* allele (see Materials and Methods). In this way, the effect of each mutation on the export of the MBP could be investigated.

Alterations in the R2 signal peptide. The precise mutational alterations in the R2 signal sequence coding region were determined by DNA sequencing (Fig. 2). Seven unique point mutations within this region were identified, each resulting in single amino acid substitutions within the hydrophobic core of the R2 signal peptide. Five of the seven alterations introduced a charged amino acid residue into the hydrophobic core; the remaining two mutations introduced proline residues into this region.

Maltose utilization by R2 signal sequence mutants. The MBP must be secreted into the periplasm to facilitate the uptake and subsequent utilization of maltose. This provides an extremely sensitive assay for the export of functional MBP. Maltose utilization by the seven isogenic R2 signal sequence mutants was analyzed, and the results are summarized in Table 1. Mutants were tested for their ability to use maltose as a sole carbon source in maltose liquid medium and on maltose minimal plates. Although their doubling times in maltose minimal medium were slightly longer, mutants CC13 and CC15 were found to utilize maltose nearly as efficiently as did isogenic *malE*$^+$ and *malEΔ12–18-R2* strains. In contrast, mutants CC17 and CC18 exhibited a greatly reduced ability to utilize maltose, and mutants CC19, CC21, and CC23 were totaly Mal$^-$.

We considered the possibility that the defect in maltose utilization observed for R2 signal sequence mutants was exacerbated by the inability to induce the synthesis of

TABLE 1. Maltose utilization by R2 signal sequence mutants

Strain	Growth on maltose minimal agar[a]		Doubling times (min) in maltose minimal liquid medium[b]	
	MalT$^+$	MalT (Con)	MalT$^+$	MalT (Con)
MC4100	+ + +	+ + +	65	ND[c]
R2	+ + +	+ + +	66	ND
CC13	+ + +	+ + +	70	70
CC15	+ + +	+ + +	75	71
CC17	+ +	+ +	135	84
CC18	+	+	>360	230
CC19	−	+	>360	240
CC21	−	−	>360	>360
CC23	−	−	>360	>360

[a] Colony size relative to that of the Mal$^+$ parental strain MC4100 following growth on maltose minimal agar plates for 48 h at 37°C. + + +, Growth was identical to that of the *malE*$^+$ strain; −, growth was not detectable even after 72 h of incubation.

[b] Cells of each strain were grown overnight in glycerol minimal medium, washed, suspended at a 1:50 dilution in maltose minimal liquid medium, and incubated at 37°C with vigorous aeration. The optical density of each culture was monitored at 600 nm, and the doubling time was calculated from the slope determined during the mid-exponential phase of growth. >360, Increase in optical density was not detected during the time course of the experiment.

[c] ND, Not done.

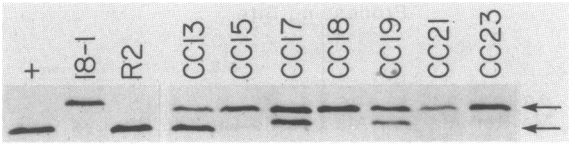

FIG. 3. Immune precipitation of MBP from R2 signal sequence mutants. Glycerol-grown, maltose-induced cells were radiolabeled for 10 min with [^{35}S]methionine. The cells were solubilized, and the MBP was immune precipitated and analyzed by SDS-PAGE and autoradiography. The corresponding mutant designations are indicated above each lane [not indicated is the fact that *malT*(Con) derivatives of mutants CC19, CC21, and CC23 were used for this experiment to elevate *mal* expression in the absence of maltose transport]. The MBP precipitated from the wild-type *malE*$^+$ strain MC4100 and the R2 mutant indicates the position of mMBP on the gel. The pMBP precipitated from the *malE18-1* mutant indicates the position of full-length pMBP. The pMBP precipitated from the various R2 signal sequence mutants migrated slightly faster than full-length precursor, because the signal peptide is 7 residues shorter. Note that only mutant CC13 produced significant amounts of mMBP.

maltose transport system components. Constitutive expression of the *mal* regulon, resulting from the introduction of the *malT*(Con) allele (11), substantially improved the utilization of maltose by strains CC17, CC18, and CC19 (Table 1). However, mutants CC21 and CC23 remained totally incapable of utilizing maltose for growth.

Analysis of MBP synthesized by R2 signal sequence mutants. The processing of precursor MBP (pMBP) to the mature species (mMBP) has, in previous studies, correlated reliably with MBP export and maltose utilization (1, 3, 5, 12, 23). Cells of each signal sequence mutant were radiolabeled with [^{35}S]methionine for 10 min, and pMBP/mMBP ratios were determined by immune precipitation, SDS-PAGE, and autoradiography, as described in Materials and Methods (Fig. 3). The MBP precipitated from the wild-type and *malEΔ12–18-R2* strains radiolabeled under these conditions was found in its mature form. MBP from each of the R2 signal sequence mutants was found primarily in the precursor form. No authentic mMBP was detectable in cell extracts of strains CC18, CC19, CC21, and CC23, correlating well with the Mal$^-$ phenotypes exhibited by these strains. The pMBP/mMBP ratios observed for strains CC13 and CC17 also were consistent with their Mal phenotypes, i.e., the greater the ability to utilize maltose, the lower the pMBP/mMBP ratios. Interestingly, this correlation did not hold true for strain CC15. In this case, only a very small amount of mMBP was detected in the precipitate, although this strain exhibits an essentially wild-type Mal$^+$ phenotype. Also, note that an MBP species migrating with an apparent molecular weight slightly higher than that of authentic mMBP was consistently precipitated from cells of strains CC17 and CC19. The intermediate MBP species observed for strain CC19 was not released by osmotic shock (20) (data not shown), indicating that it was not localized in the periplasm. Such proteolytic breakdown products of internalized pMBP have been encountered previously (3, 5). Localization studies of MBP synthesized by strain CC17 are presented in the accompanying article (15).

Kinetics of MBP export. The rate of MBP maturation in the seven R2 signal sequence mutants was determined by pulse-chase analysis. Strains were pulse-labeled for 15 s with [^{35}S]methionine and then incubated for various periods in a chase solution containing unlabeled methionine. The chase periods were terminated by the addition of an equal volume of ice-cold 10% trichloroacetic acid, and the labeled MBP species present at each chase point were analyzed by immune precipitation, SDS-PAGE, and autoradiography (Fig. 4). As found previously (3, 23), the wild-type and R2 signal peptides mediated comparable, very rapid rates of MBP export; only small amounts of pMBP were detected at the early time points. In contrast, no mMBP was detected at the earliest chase point for any of the R2 signal sequence mutants. For only one of the mutants exhibiting a strong Mal$^+$ phenotype, strain CC13, was substantial MBP maturation observed at later chase points. After 20 min of chase, approximately 70% of the radiolabeled MBP migrated as mMBP. At no chase point could mMBP be detected for the strong signal sequence mutants CC18, CC19, CC21, and CC23. Excluding the apparent pMBP breakdown product observed for strain CC19, all MBP detected in these strains migrated as pMBP. For strains CC15 and CC17, a small amount of authentic mMBP accumulated at the later chase times. However, pMBP and an intermediate MBP species clearly represented the great majority of radiolabeled MBP present.

Effect of extragenic suppressors on R2 signal sequence mutations Numerous extragenic suppressor mutations (designated *prl*) that can restore, with varying efficiencies, the export of proteins with defective signal peptides have previously been isolated and characterized (for a review, see reference 25). Three extragenic suppressors, *prlA4* (12, 13), *prlA402* (2), and *prlD2* (23), were individually analyzed for their effect on both maltose utilization and MBP export when introduced into strains harboring R2 signal sequence mutations. These results are summarized in Table 2. In general, the degree of suppression observed was dependent on both

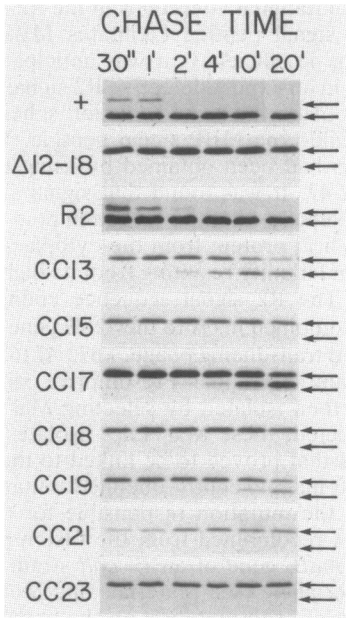

FIG. 4. Kinetics of MBP export in wild-type and various mutant strains. Glycerol-grown, maltose-induced cells were pulse-labeled for 15 s with [^{35}S]methionine, and the chase was initiated by addition of unlabeled methionine in large excess. At the time indicated above each lane, the case was terminated by the addition of trichloroacetic acid. Subsequently, the trichloroacetic acid precipitates were solubilized, and the MBP was immune precipitated and analyzed by SDS-PAGE and autoradiography. For each gel, the positions of intact pMBP and mMBP are indicated by arrows at right.

the choice of suppressor and the severity of the export defect manifested by the mutant signal peptide. For a variety of *malE* and *lamB* signal sequence mutations, the *prlA402* allele is the strongest extragenic suppressor obtained to date (2). The effect of this suppressor mutation on the pMBP/mMBP ratio of the various R2 signal sequence mutants is shown in Fig. 5. The following may be noted from Fig. 5 and Table 2. (i) For mutant CC15, the pMBP/mMBP ratio was not affected by the presence of any *prl* suppressor mutation, including *prlA402* (see Fig. 3 for comparison). (ii) Four of the R2 signal sequence mutations were fairly efficiently suppressed by *prlA402*. This is clearly indicated by the relative increase in authentic mMBP observed in precipitates from mutants CC13, CC17, CC18, and CC19. (iii) Suppression of mutant CC21 by the two *prlA* alleles tested was very inefficient. In fact, such suppression was detectable only by the more sensitive maltose utilization assay; no obvious change in the pMBP/mMBP ratio of this mutant was discernible. Suppression of CC21 by *prlD2* was not detectable by either assay. (iv) Mutant CC23 was not suppressed by any of the extragenic *prl* mutations tested.

DISCUSSION

On the basis of conclusions made from detailed comparisons of a number of procaryotic and eucaryotic signal peptides (21, 28), the functional hydrophobic core of the wild-type MBP signal peptide probably is composed of residues 9 through 20. Single amino acid substitutions that adversely affect the export competence of this structure have been confined to the region between positions 10 and 19 (7). The removal of residues 12 through 18 from the central core region (*malEΔ12–18*) results in an almost total block in MBP export. It was proposed that the core structure that remains is too short to promote initiation of MBP secretion, even if the residues making up the signal peptidase recognition site could serve in this capacity (3). However, the substitution of Leu for Arg at residue 8, as occurred in the R2 mutant, extends the truncated core into the hydrophilic segment a sufficient distance to regenerate a fully functional signal peptide (3).

TABLE 2. Suppression of R2 signal sequence mutations by *prl* alleles

Strain	*prlA4*		*prlA402*		*prlD2*	
	Growth[a]	Processing[b]	Growth[a]	Processing[b]	Growth[a]	Processing[b]
CC13	ND[c]	+	ND[c]	+	ND[c]	+
CC15	ND[c]	ND[d]	ND[c]	ND[d]	ND[c]	ND[d]
CC17	+	+	+	+	+	+
CC18	+	+	+	+	+	+
CC19	+	+	+	+	+	+
CC21	+	−	+	−	−	−
CC23	−	−	−	−	−	−

[a] Suppression was scored by observing the color reaction of mutant colonies on maltose tetrazolium indicator plates and by the colony size on maltose minimal agar plates. +, Suppression was observed.

[b] Suppression was indicated by changes in the pMBP/mMBP ratio (Fig. 5). +, Suppression was observed.

[c] ND, Suppression indicated by changes in growth phenotype would not be detectable for mutants CC13 and CC15, since these strains utilize maltose as efficiently as does a wild-type Mal$^+$ strain.

[d] ND, Suppression indicated by changes in pMBP/mMBP ratio would not be detectable for mutant CC15, since the pMBP secreted by this strain is very inefficiently processed. See Discussion and reference 15.

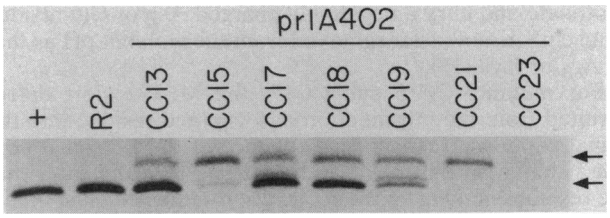

FIG. 5. Immune precipitation of MBP from R2 signal sequence mutants harboring the *prlA402* suppressor allele. The corresponding mutant designations are indicated above each lane. See the legend to Fig. 3 for further details. Owing to the lack of suppression of mutants CC21 and CC23 by *prlA402* and hence the inability of maltose to induce *mal* regulon expression, much less MBP was seen in precipitates obtained from these cells.

In this study, we obtained a number of alterations in the R2 signal peptide that resulted in an export-defective MBP. In two instances, a proline residue was introduced into the hydrophobic core. These mutants are similar to the *malE10-1* mutant isolated previously (7); the negative effect of the proline residue presumably is to disrupt the α-helical conformation through the core region (8, 14). A significant difference was observed in the export competence of the MBP synthesized by mutants CC13 and CC19. When Pro was introduced at residue 21 (CC13), the majority of MBP was exported and processed, although at a rate considerably slower than that in the wild type, and the strain exhibited a fully Mal$^+$ phenotype. In marked contrast, when Pro was introduced at residue 8 (CC19), periplasmic mMBP could not be detected, and the strain could only inefficiently utilize maltose for growth if a *malT*(Con) allele were present. In the latter case, the helix-breaking property of the Pro residue was probably considerably amplified by its close proximity to the Gly at residue 6, which is also a strong helix breaker (10). This was reinforced by the recent isolation of a Mal$^+$ revertant of CC19 in which Cys was substituted for Gly at residue 6 (J. Puziss and J. Fikes, unpublished data). Emr and Silhavy (14) previously demonstrated that the close proximity of Gly and Pro in the LamB protein signal peptide results in a major export defect. The results presented here further emphasize the importance of the secondary structure through the core region.

The introduction of charged residues into the R2 signal peptide hydrophobic core also adversely affected MBP export, as expected. For two of the mutants, CC21 and CC23, the MBP was rendered totally export defective and the strains were unable to utilize maltose as a carbon source, even if a *malT*(Con) allele were present. In contrast, none of the *malE* signal sequence mutants previously obtained, including *malEΔ12–18*, exhibited a totally Mal$^-$ phenotype. Thus, it appears that the shorter R2 signal peptide, when compared with the wild-type structure, is considerably easier to inactivate totally. It also is clear from this analysis that the introduction of a negatively charged residue into the hydrophobic core has a slightly less deleterious effect on signal peptide function than the introduction of a positively charged residue (e.g., compare mutant CC18 with mutants CC21 and CC23). Such a finding further suggests that the hydrophobic core directly interacts with the nonpolar environment of the lipid bilayer, where it is slightly easier to

protonate and bury a negatively charged Asp or Glu residue whose pK is not as far removed from the ambient pH as that of Arg or Lys (16).

For mutants CC15 and CC17, the MBP export defect resulted from the introduction of a charged residue into the signal peptidase recognition sequence of the R2 signal peptide. This is an important finding, because it indicates that the residues making up the recognition sequence must in this case also be considered part of the functional hydrophobic core. It has been suggested that the recognition sequence is in part designated by the termination of the core and follows a predicted β-hairpin turn some five to six residues prior to the cleavage site (21). For the wild-type MBP, a β-turn is predicted at residue 21 (Ser) of the signal peptide (21). According to this model, the cleavage site is exposed on the external side of the membrane, where it is accessible to signal peptidase. However, in the R2 signal peptide, it could be that the hydrophobic core and signal peptidase recognition sequence significantly overlap without having any obvious effect on pMBP export or processing.

In this study, *prlA*- and *prlD*-mediated suppression of export defects in the R2 signal peptide also were investigated. The *prlA* and *prlD* products are believed to be components of the normal cellular secretion machinery that directly interact with the signal peptide during an early step in the export process (2, 12, 13, 23). These Prl proteins can apparently be altered in such a manner as to restore the export of proteins with defective signal peptides. It was found that most of the mutations altering the R2 signal peptide were suppressed to some degree by the different *prl* alleles tested. However, the CC23 mutation was not detectably suppressed by any of these *prl* alleles. This was somewhat unexpected, since the signal sequence alterations resulting from the CC23 mutation and *malE19-1* are identical (Fig. 1 and 2) and the latter is very efficiently suppressed by both *prlA4* and *prlA402* (2, 12). Furthermore, these *prlA* mutations had previously been shown to restore the export of proteins with extremely defective signal peptides, including a mutant LamB protein missing nearly its entire hydrophobic core (12, 13). Thus, the CC23 mutation not only totally inactivates the R2 signal peptide, but also eliminates whatever residual signal peptide activity is required for *prlA*-mediated protein export. Mal^+ revertants of the CC23 mutant are currently being isolated and characterized. Such an analysis should help to define the absolute minimal requirements for a functional MBP signal peptide.

Finally, it was quite intriguing that only minute quantities of mMBP were detected in immune precipitates obtained from cells of the CC15 mutant, although this strain exhibited an essentially wild-type Mal^+ phenotype. Even in pulse-chase studies, very little mMBP was seen to accumulate at the later chase times. In this mutant, the first residue of the consensus Ala-X-Ala processing sequence for signal peptidase was converted to an Asp. It could be that the CC15 mutant was exporting considerable quantities of functional MBP to the periplasm, although pMBP maturation was inhibited by the alteration in the processing site. Likewise, for mutant CC17, a large quantity of an MBP species that migrated on SDS-PAGE only slightly slower than wild-type mMBP was detected in immune precipitates. In this case, the signal peptide alteration was in the middle residue of the consensus processing sequence. While it might not be expected that this alteration would affect pMBP processing by signal peptidase (21, 26, 28), note that there is also a potential Ala-X-Ala processing sequence at residues 22 to 24 of the MBP signal peptide. It seemed possible that exported pMBP was processed aberrantly in the CC17 mutant, resulting in the production of a periplasmic MBP species that was two residues larger than wild-type mMBP. Thus, the export of MBP in mutants CC15 and CC17 was investigated in considerable detail, as reported in the accompanying article by Fikes and Bassford (15).

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LITERATURE CITED

1. **Bankaitis, V. A., and P. J. Bassford, Jr.** 1984. The synthesis of export-defective proteins can interfere with normal protein export in *Escherichia coli*. J. Biol. Chem. **259**:12193–12200.
2. **Bankaitis, V. A., and P. J. Bassford, Jr.** 1985. Proper interaction between at least two components is required for efficient export of proteins to the *Escherichia coli* cell envelope. J. Bacteriol. **161**:169–178.
3. **Bankaitis, V. A., B. A. Rasmussen, and P. J. Bassford, Jr.** 1984. Intragenic suppressor mutations that restore export of maltose binding protein with a truncated signal peptide. Cell **37**:243–252.
4. **Bankaitis, V. A., J. P. Ryan, B. A. Rasmussen, and P. J. Bassford, Jr.** 1985. The use of genetic techniques to analyze protein export in *Escherichia coli*. Curr. Top. Membr. Transp. **24**:105–150.
5. **Bassford, P., and J. Beckwith.** 1979. *Escherichia coli* mutants accumulating the precursor of a secreted protein in the cytoplasm. Nature (London) **277**:538–541.
6. **Bassford, P. J., Jr., T. J. Silhavy, and J. R. Beckwith.** 1979. Use of gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. J. Bacteriol. **139**:19–31.
7. **Bedouelle, H., P. J. Bassford, Jr., A. V. Fowler, I. Zabin, J. Beckwith, and M. Hofnung.** 1980. Mutations which alter the function of the signal sequence of the maltose binding protein of *Escherichia coli*. Nature (London) **285**:78–81.
8. **Bedouelle, H., and M. Hofnung.** 1981. Functional implications of secondary structure and analysis of wild-type and mutant signal peptides, p. 309–343. *In* D. Oxender (ed.), Membrane transport and neuroreceptors. Alan R. Liss, Inc., New York.
9. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu. J. Mol. Biol. **104**:541–555.
10. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47**:251–276.
11. **Debarbouille, M., H. A. Shuman, T. J. Silhavy, and M. Schwartz.** 1978. Dominant constitutive mutations in *malT*, the positive regulator gene of the maltose regulon in *Escherichia coli*. J. Mol. Biol. **124**:359–371.
12. **Emr, S. D., and P. J. Bassford, Jr.** 1982. Localization and processing of outer membrane and periplasmic proteins in *Escherichia coli* strains harboring export-specific suppressor mutations. J. Biol. Chem. **257**:5852–5860.
13. **Emr, S. D., S. Hanley-Way, and T. J. Silhavy.** 1981. Suppressor mutations that restore export of a protein with a defective signal sequence. Cell **23**:79–88.
14. **Emr, S. D., and T. J. Silhavy.** 1983. Importance of secondary structure in the signal sequence for protein secretion. Proc. Natl. Acad. Sci. USA **80**:4599–4603.
15. **Fikes, J. D., and P. J. Bassford, Jr.** 1986. Export of unprocessed precursor maltose-binding protein to the periplasm of *Escherichia coli* cells. J. Bacteriol. **169**:2352–2359.
16. **Honig, B. H., and W. C. Hubbell.** 1984. Stability of "salt bridges" in membrane proteins. Proc. Natl. Acad. Sci. USA **81**:5412–5416.
17. **Ito, K., P. J. Bassford, Jr., and J. R. Beckwith.** 1981. Protein localization in *E. coli*: is there a common step in the secretion of periplasmic and outer membrane proteins? Cell **24**:707–717.

18. **Michaelis, S., and J. Beckwith.** 1982. Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. Annu. Rev. Microbiol. **36**:435–464.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Neu, H. C., and L. A. Heppel.** 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685–3692.
21. **Perlman, D., and H. O. Halvorson.** 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. **167**:391–409.
22. **Rasmussen, B. A., and P. J. Bassford, Jr.** 1985. Both linked and unlinked mutations can alter the intracellular site of synthesis of exported proteins of *Escherichia coli*. J. Bacteriol. **161**:258–264.
23. **Ryan, J. P., and P. J. Bassford, Jr.** 1985. Post-translational export of maltose-binding protein in *Escherichia coli* strains harboring *malE* signal sequence mutations and either *prl*$^+$ or *prl* suppressor alleles. J. Biol. Chem. **260**:14832–14837.
24. **Ryan, J. P., M. C. Duncan, V. A. Bankaitis, and P. J. Bassford, Jr.** 1986. Intragenic reversion mutations that improve export of maltose-binding protein in *Escherichia coli malE* signal sequence mutants. J. Biol. Chem. **261**:3389–3395.
25. **Silhavy, T. J., S. A. Benson, and S. D. Emr.** 1983. Mechanisms of protein localization. Microbiol. Rev. **47**:313–344.
26. **von Heijne, G.** 1983. Patterns of amino acids near signal sequence cleavage sites. Eur. J. Biochem. **116**:17–21.
27. **von Heijne, G.** 1984. How signal sequences maintain sequence specificity. J. Mol. Biol. **173**:243–251.
28. **von Heijne, G.** 1985. Structural and thermodynamic aspects of the transfer of proteins into and across membranes. Curr. Top. Membr. Transp. **24**:151–179.
29. **von Heijne, G.** 1986. Signal sequences: the limits of variation. J. Mol. Biol. **184**:99–105.
30. **von Heijne, G.** 1986. Net N-C charge imbalance may be important for signal sequence function in bacteria. J. Mol. Biol. **192**:287–290.